REMARKS

Status of the Claims

Claims 1-10 are currently pending in the application. Claims 1-3 stand rejected. Claims 4-8 are withdrawn as being drawn to a non-elected invention. Claims 1-3 have been amended without prejudice or disclaimer. New claims 9 and 10 have been added. No new matter has been added by way of the present amendments. Specifically, the amendments to claims 1-3 are to clarify that the strains claimed are "biologically pure" cultures, as suggested by the Examiner. The term "biologically pure" is intended to define the invention consistent with the explicit and implicit written description of the disclosure as filed. The term excludes products found in nature and requires manipulation by man (directly or indirectly).

Furthermore, claim 3 has been amended to recite the full name of the biological deposit number of the strain and is supported by the specification at, for instance, page 6. New claim 9 is supported by the specification at, for instance, page 4, lines 1-9 and lines 22-28, and Example 3. Support for new claim 10 is provided by claims 1-3. Reconsideration is respectfully requested.

Rejections Under 35 U.S.C. § 101

Claims 1-3 stand rejected under 35 U.S.C. § 101 because they allegedly are directed to non-statutory subject matter. (*See*, Office Action of November 15, 2007, at page 2, hereinafter, "Office Action"). Applicants traverse the rejection as set forth herein.

The Examiner states that the claims encompass naturally occurring bacteria or products of nature. The Examiner recommends amending the claims to recite "A biologically pure culture of a microorganism ..." Applicants have amended claims 1-3 as suggested by the Examiner.

Reconsideration and withdrawal of the rejection of claims 1-3 are respectfully requested.

Rejections Under 35 U.S.C. § 112, Second Paragraph

Claim 3 stands rejected under 35 U.S.C. § 112, second paragraph, for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. (*See*, Office Action, at page 3). Applicants traverse the rejection as set forth herein.

The Examiner states that claim 3 recites an arbitrary nomenclature for the claimed strain. The Examiner states that this should be amended to instead recite the actual deposit identifier instead of the arbitrary name used by Applicants.

Although Applicants do not agree that claim 3 is indefinite, to expedite prosecution, claim 3 has been amended to recite, in part, "*Rhodococcus equi* strain TB-60-DMZ 16175." Thus, claim 3 now recites the full name of the strain as deposited at the DSMZ. This amendment is supported at least at page 6 of the present specification.

Reconsideration and withdrawal of the indefiniteness rejection of claim 3 are respectfully requested.

Rejections Under 35 U.S.C. § 112, First Paragraph

Claim 3 stands rejected under 35 U.S.C. § 112, first paragraph, for failing to comply with the enablement requirement. (*See*, Office Action, at pages 3-5). Applicants traverse the rejection as set forth herein.

The Examiner states that the deposit made by Applicants does not satisfy the requirements of the Budapest Treaty on Biological Deposits and therefore further assurances are required from Applicants.

Applicants respectfully direct the Examiner's attention to MPEP 2405 which provides a current list of all authorized Biological Depository Authorities. Said list includes the German depository institution Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures, DSMZ or DMZ). (*See*, MPEP § 2405, page 2400-9, righthand column). Additional response to this rejection will follow shortly.

Rejections Under 35 U.S.C. § 102(a)

Claims 1-3 stand rejected under 35 U.S.C. § 102(a) as being anticipated by Adachi et al., *Japan Soc. For Biosci., Biotech., Agrochem. 2003 Nendo*, 2003 (hereinafter, "Adachi et al."). (*See*, Office Action, at page 5). Applicants traverse the rejection as set forth herein.

Applicants submit herewith a verified English language translation of their priority document JP 2003/55421, which has a filing date of March 3, 2003. By this submission, Applicants have removed Adachi et al., which was published on March 5, 2003, as prior art.

Reconsideration and withdrawal of the anticipation rejection of claims 1-3 are respectfully requested.

Rejections Under 35 U.S.C. §§ 102(b) or 103(a), Anticipation or Obviousness

Claims 1-3 stand rejected under 35 U.S.C. § 102(b) as anticipated by, or in the alternative, under 35 U.S.C. § 103(a) as obvious over Ohshiro et al., *Appl. Microbiol. Biotechnol.*, 48:546-548, 1997 (hereinafter, "Ohshiro et al.") in view of ATCC Catalog. (*See*, Office Action, at pages 5-6). Applicants traverse the rejection as set forth herein.

The Examiner states that Ohshiro et al. disclose a *Mycobacterium* strain F-96 and other strains, which have the ability to degrade urethane bonds. The Examiner notes that strains of *Mycobacterium*, *Arthrobacter*, *Brevibacterium*, and *Nocardia* were all recently re-classified as *Rhodococcus*. The Examiner states that Ohshiro et al. discloses a Mycobacterium strain F-96 and other strains, at least one of which appears to be identical to the claimed Rhodococcus strain or a mutant thereof. The Examiner further states that many strains of *Mycobacterium*, *Arthrobacter*, *Brevibacterium* and *Nocardia* have been reclassified as *Rhodococcus*.

However, the strains mentioned in Ohshiro et al. do not belong to *Rhodococcus*, as explained in further detail as follows.

<u>F-96</u>

Strain F-96 is not identified as *Mycobacterium*. Enclosed herewith is a copy of Matsumura et al. (1985b), *Agric. Biol. Chem.*, 49:3643-3645, 1985 (Exhibit A), which is cited in Ohshiro et al. at page 548, left column, bottom of page. In Matsumura et al., there are no data identifying the strain F-96, and therefore there is no disclosure or suggestion that strain F-96 belongs to *Mycobacterium*.

Corynebacterium aquaticum IFO 12154

Corynebacterium aquaticum IFO 12154 corresponds to ATCC14665, as shown in the attached printout from the ATCC website (Exhibit B). Exhibit B is a copy of the IFO catalogue. As reflected therein, *Corynebacterium aquaticum* has been reclassified as *Leifsonia aquatica*, as also shown at the following website URL:

www.atcc.org/common/catalog/numSearch/numResults.cfm?atccNum=14665.

This strain is therefore a type strain, and does not belong to *Rhodococcus*.

Arthrobacter unreafaciens IFO 12140

Arthrobacter unreafaciens IFO 12140 corresponds to ATCC7562, as shown in the attached single page printout of Appendix C. This strain is a type strain, and therefore the strain also is not *Rhodococcus*.

Arthrobacter sulfurens IFO 12648

Arthrobacter sulfurens IFO 12648 corresponds to ATCC14756, as shown in the attached single page printout of Exhibit D, and is identified as *Serratia marcescens*.

Brevibacterium incertum IFO 12145

Brevibacterium incertum IFO 12145 corresponds to ATCC8363, as shown in the attached single page printout of Exhibit E. This strain has been reclassified as *Desemzia incerta*, as shown in the website URL:

www.atcc.org/common/catalog/numSearch/numResults.cfm?atccNum=8363

This strain is also a type strain, and therefore does not belong to *Rhodococcus*.

Brevibacterium stationis IFO 12144

Brevibacterium stationis IFO 12144 corresponds to ATCC14403, as shown in the attached Exhibit F. This strain is a type strain, and therefore also does not belong to *Rhodococcus*, as also shown in the website URL:

atcc.org/common/catalog/numSearch/numResults.cfm?atccNum=14403

Brevibacterium fuscum IFO 12127

Brevibacterium fuscum IFO 12127 corresponds to CCEB277 or JCM1488, as shown in the attached Exhibit G, and as reflected in the website URL:

nbrc.nite.go.jp/jscc/idb/strains?Snt=0&Sn=Brevibacterium+fuscum

This strain has not been reported as *Rhodococcus*.

Cellulomonas flavigena IFO 3754

Cellulomonas flavigena IFO 3754 corresponds to ATCC482, as shown in the attached Exhibit H. This strain is a type strain, and therefore cannot belong to *Rhodococcus*. (www.atcc.org/common/catalog/numSearch/numResults.cfm?atccNum=482).

Nocardia globerula IFO 13510

Nocardia globerula IFO 13510 corresponds to ATCC21292, as shown in the attached Exhibit I. This strain has not been reported as *Rhodococcus* (www.atcc.org/common/catalog/numSearch/numResults.cfm?atccNum=21292).

These facts clearly indicate that none of the strains disclosed in Ohshiro et al. belong to *Rhodococcus*. Further, there is no suggestion or support in the Ohshiro et al. disclosure to conclude that the strains mentioned in Ohshiro et al. are related to *Rhodococcus*. Therefore, the strain recited in claim 3 is obviously different from the strains disclosed in Ohshiro et al.

Urethane compounds, represented by the general formula R1-NHCOO-R2, are classified based on types of substitutions. For instance, see the Introduction section of the attached reference, Exhibit J, "Isolation of a bacterium that degrades urethane compounds and characterization of its urethane hydrolase." Urethane bonds in polyurethane are mainly N-aryl acylcarbamate, in which the benzene ring is bonded to NH.

However, Ohshiro et al. disclose strains which can only degrade boc-amino acid (alkyl-carbamate), and Z-amino acid (N-acylarylcarbamate),. Ohshiro et al. do not disclose strains which have the ability to degrade N-aryl acylcarbamate. Further, as mentioned in the Introduction section of Exhibit I, three types of hydrolases which are known to cleave a urethane

bond have been studied. Most of the urethane compounds previously studied are type I and type II substrates, whereas urethane groups in the most common type of polyurethane are type III substrates.

This means that the disclosures in Ohshiro et al. directed to the microorganisms which can degrade Boc-L-Met can not lead one of ordinary skill in the art to believe, with any expectation of success, that these strains could also degrade the urethane bonds in polyurethane.

Thus, reconsideration and withdrawal of the anticipation or obviousness rejections of claims 1-3 are respectfully requested.

CONCLUSION

If the Examiner has any questions or comments, please contact Thomas J. Siepmann, Ph.D., Registration No 57,374, at the offices of Birch, Stewart, Kolasch & Birch, LLP.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to our Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under § 1.17; particularly, extension of time fees.

Dated: March 17, 2008

Respectfully submitted,

By #43575

Marc S. Weiner
Registration No.: 32,181
BIRCH, STEWART, KOLASCH & BIRCH, LLP
8110 Gatehouse Road
Suite 100 East
P.O. Box 747
Falls Church, Virginia 22040-0747
(703) 205-8000
Attorney for Applicants

Attachments: Verified English Language Translation of JP 2003/55421
Exhibit A - Matsumura et al. (1985b), *Agric. Biol. Chem.*, 49:3643-3645, 1985
Exhibit B - Copy of ATCC website concerning ATCC14665
Exhibit C – Copy of ATCC website concerning ATCC7562
Exhibit D – Copy of ATCC website concerning ATCC14756
Exhibit E – Copy of ATCC website concerning ATCC8363
Exhibit F – Copy of ATCC website concerning ATCC14403
Exhibit G – Copy of NBRC website concerning CCEB277 or JCM1488
Exhibit H – Copy of ATCC website concerning ATCC482
Exhibit I – Copy of ATCC website concerning ATCC21292
Exhibit J – Isolation of a bacterium that degrades urethane compounds and characterization of its urethane hydrolase.

Exhibit A

Agric. Biol. Chem., **49** (12), 3643~3645, 1985

Short Communication

A Novel Enzyme, N^{α}-Benzyloxy-carbonyl Amino Acid Urethane Hydrolase IV

Eiko MATSUMURA, Takashi SHIN,*,† Sawao MURAO,*,† Minoru SAKAGUCHI and Tatsu KAWANO

Laboratory of Microbial Products, Osaka College of Pharmacy, Matsubara, Osaka 580, Japan
**Department of Agricultural Chemistry, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 591, Japan*

Received September 2, 1985

In the course of studies on the deprotection reaction of N^{α}-protecting group of acylamino acids by microorganism, we found a new enzyme, N^{α}-benzyloxycarbonyl(Z) amino acid urethane hydrolase (urethane hydrolase), that hydrolyzes urethane bonds in Z-amino acids. We have reported urethane hydrolases I,[1~3] II,[4] and III,[5] which catalyze the hydrolysis of Z-Gly, -Ala (urethane hydrolase I), Z-Arg (urethane hydrolase II), and Z-Gly, -Ala, -Ser (urethane hydrolase III). These enzymes were all inactive on N^{α}-*t*-butoxycarbonyl(Boc) amino acids, even though the Boc group also has a urethane type protecting function. Through subsequent investigations, we found a microorganism, strain F-96, having high hydrolytic activity on Boc-amino acids. This is the first finding of an effective enzyme for Boc-moieties of acyl-amino acids. We report the purification and enzymological properties in this paper.

The enzyme activity was measured by the photometric ninhydrin method,[6] measuring the amount of released amino groups. The reaction was done in a mixture containing 1 μmol of Z-Asp, 1 μmol of Co^{2+}, and the enzyme in a total volume of 1.0 ml, buffered with 0.1 M AcOH–AcONa buffer (pH 5.8) at 37°C.

Strain F-96 was isolated from soil samples collected at Takaoka-cho, Miyazaki prefecture. The medium for the production of the enzyme consisted of 2% meat extract and 1% polypepton in tap water. The pH of the medium was adjusted to 7.0 with 2 N NaOH before autoclaving. The maximum enzyme production was attained by the cultivation at 27°C for about 3 days in a shaking flask. The cells were harvested from 25 liters of cultured medium by centrifugation (6000 rpm, 10 min) and made into a cell-free extract by disrupting the washed cells with sea sand in a mortar. The enzyme was purified by ammonium sulfate fractionation (50~80% saturation), treatment with streptomycin sulfate (protein-to streptomycin sulfate ratio of 1 to 1), and column chromatography on DEAE-Toyopearl 650M (1st), Sephacryl S-300, Toyopearl HW-55S and DEAE-Toyopearl 650M (2nd). The overall purification of the enzyme was 1780-fold with an activity yield of about 1.2%. The final preparation of the enzyme was electrophoretically homogeneous as judged from the protein band stained with Coomassie Brilliant Blue-250.

The molecular weight of the enzyme was estimated to be 39,000 by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and 116,000 by gel filtration on Toyopearl HW-55S. The optimum temperature for the activity of this enzyme was 55°C in 10-min reactions. The highest activity was found at pH 5.1 in 0.01 M AcOH–AcONa buffer. The enzyme was stable for 17 hr in the range between pH 6.0 and 7.0 at 37°C. The isoelectric point of the purified enzyme was 3.31 by flat bed isoelectrofocusing. The enzyme was completely inhi-

† Present address: *Department of Applied Microbial Technology, The Kumamoto Institute of Technology, Kumamoto 860, Japan.*

Abbreviations: Abbreviated designations of amino acids and their derivatives obey the tentative rules recommendes by the IUPAC-IUB commission on Biochemical Nomenclature (1965). Except when specified, the constituent amino acids were all in the L-configuration.

TABLE I. THE RATE OF HYDROLYSIS OF VARIOUS SUBSTRATES

The reaction was done at 55°C in a mixture containing 1.0 μmol of substrate, 1.0 μmol of Co^{2+}, and 50 μl of the enzyme (0.2 μg of protein) in a total volume of 1.0 ml, buffered with 0.1 M AcOH–AcONa buffer (pH 5.8). Released amino acids were measured by the photometric ninhydrin method.

	Reaction rate (μmol/min/mg*)		Reaction rate (μmol/min/mg*)		Reaction rate (μmol/min/mg*)
Z-Asp	6.0	Z-D-Asp	0	Bz(*p*-OH)-Asp	2.5
-Asn	2.3	-D-Glu	0	Bz-Glu	6.0
-Glu	4.1	-D-Gln	0	-Leu	7.4
-Gln	2.4	-Asn-OBu^t	0	Bz(*p*-NH_2)-Glu	1.0
-Gly	1.8	-Leu-NH_2	0		
-Ala	3.8			Ac-Asp	0.7
-Val	1.4	Boc-Asp	5.5	-Glu	1.8
-Leu	27.8	-Gln	2.1	-Leu	11.1
-Ile	0.3	-Leu	21.7	-Pro	0
-Ser	2.7	-Gly	5.3	-Arg	0
-Thr	0.3	-Ala	4.8	-Lys	0
-Cys(Bzl)	3.8	-Val	1.5		
-Met	4.9	-Met	2.4	Methyl-Asp	0
-His	0.3	-Trp	0	Amidino-Asp	0
-Phe	0.9	-Pro	0	Tosyl-Glu	0
-Tyr	0.3	-Arg	0		
-Trp	0	-Lys	0	Folic acid	0
-Pro	0	-β-Ala	0	Methotrexate	0
-Arg	0	-D-Leu	0	Casein	0
-Lys	0	-D-Met	0		
-β-Ala	0				

* Protein was measured spectrophotometrically by the absorbance at 224~233.3 nm, assuming that the value of $(\Delta A_{224} \sim A_{233.3}) \times 210$ is the concentration (μg/ml) of protein.[10]

bited by disodium ethylenediamine tetraacetate and strongly inhibited by *p*-chloromercuribenzoate and iodoacetate at the concentration of 1 mM. The purified enzyme required divalent cations (Co^{2+} or Zn^{2+}) for its activity, and the optimum concentration of Co^{2+} was about 1 mM.

The substrate specificity of the purified enzyme is summarized in Table I. Z-Leu was most rapidly hydrolyzed, and a number of Z-amino acids such as Z-Asp, -Met, -Glu, and -Ala were also hydrolyzed by the enzyme. The data show that the enzyme from strain F-96 has broadly selective amino acid specificity, in contrast with urethane hydrolase I, II, and III having high selectivity for amino acid residues;[3~5] characteristically it can hydrolyze Boc-amino acids. The rate of hydrolysis of Boc-Leu was 80% of that of Z-Leu. This result is very interesting, considering that urethane hydrolases I, II, or III cannot hydrolyze any Boc-amino acid. In addition, this enzyme hydrolyzed N^α-acetyl (Ac)-Leu at 40% of the reaction rate of Z-Leu. However, urethane hydrolases I and III could not hydrolyze Ac-amino acids at all[3,5] and urethane hydrolase II had some activity toward Ac-Arg.[4] The following three observations show that the enzyme from strain F-96 has quite similar substrate specificity to urethane hydrolases I, II, and III. (a) N^α-Methyl-Asp, N^α-Amidino-Asp, and N^α-Tosyl-Glu were all unaffected by the enzyme, suggesting that the binding type between the N^α-protecting group and the amino acid should be a urethane bond or acyl bond. (b) The blocking of the carboxyl group of the amino acid residue as in Z-Leu-NH_2 and Z-Asn-OBu^t inhibited the catalytic activity of this enzyme, so the free carboxyl group of amino acids is required for its catalytic ac-

tivity. (c) The enzyme was inactive toward Z-D-amino acids such as Z-D-Asp, Z-D-Glu, and Boc-D-Leu, therefore, the activity of this enzyme seems to be specific for the L-isomers.

The folate-hydrolyzing enzyme from *Crithidia fasciculata*[7] and bacterial carboxypeptidases G[8] and G_1[9] hydrolyze some Z-amino acids, as well as folic acid and methotrexate. However, this enzyme failed to hydrolyze folic acid and methotrexate.

From the novel feature of substrate specificity, the enzyme from strain F-96 is suggested to be a new type of urethane hydrolase. Thus, we have tentatively named the enzyme urethane hydrolase IV.

A more detailed description of this work will be reported elsewhere.

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1) S. Murao, E. Matsumura, T. Shin and T. Kawano, *Agric. Biol. Chem.*, **48**, 1673 (1984).
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10) W. E. Groves, F. C. Jr. Davis and B. H. Sells, *Anal. Biochem.*, **22**, 195 (1968).

Exhibit B

Product Description

Before submitting an order you will be asked to read and accept the terms and conditions of ATCC's Material Transfer Agreement or, in certain cases, an MTA specified by the depositing institution.

Customers in Europe, Australia, Canada, China, Hong Kong, India, Japan, Korea, Macau, Mexico, New Zealand, Singapore, and Taiwan, R.O.C. must contact a local distributor for pricing information and to place an order for ATCC cultures and products.

Bacteria

ATCC® Number:	14665™ Order this item	**Price:**	$185.00
Organism:	*Leifsonia aquatica* (ex Leifson) Evtushenko et al.; deposited as *Corynebacterium aquaticum* Leifson		
Designations:	150 [DSM 20146; JCM 1368; NCIB 9460; VKM Ac-1400]	**Isolation:**	distilled water, Illinois
Depositor:	E Leifson		
Biosafety Level:	1	**Shipped:**	freeze-dried
Growth Conditions:	ATCC medium 3: Nutrient agar or nutrient broth Temperature: 26.0C		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		

Related Products

Cross References:	GenBank: M16169: C.aquaticum 5S rRNA.
Type Strain:	yes
References:	6344: Leifson E. The bacterial flora of distilled and stored water. III. New species of the genera Corynebacterium, Flavobacterium, Spirillum and Pseudomonas. Int. Bull. Bacteriol. Nomencl. Taxon. 12: 161-170, 1962. 48864: Evtushenko LI, et al. Leifsonia poae gen. nov., sp. nov., isolated from nematode galls on Poa annua, and reclassification of 'Corynebacterium aquaticum' Leifson 1962 as Leifsonia aquatica (ex Leifson 1962) gen. nov., nom. rev., comb. nov. and Clavibacter xyli Davis et al. 1984 with two subspecies as Leifsonia xyli (Davis et al. 1984) gen. nov., comb. nov.. Int. J. Syst. Evol. Microbiol. 50: 371-380, 2000. PubMed: 10826825

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Bacteria

ATCC® Number:	**7562™** [Order this item] **Price:** **$225.00**
Organism:	*Arthrobacter ureafaciens* (Krebs and Eggleston) Clark; deposited as *Corynebacterium creatinovorans* Dubos and Miller
Designations:	NC [NCIB 7811]
Depositor:	RJ Dubos
Biosafety Level:	1 **Shipped:** freeze-dried
Growth Conditions:	ATCC medium 3: Nutrient agar or nutrient broth Temperature: 26.0C
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
	Related Products
Cross References:	GenBank: AF214782: Arthrobacter ureafaciens RecA protein (recA) gene, partial cds.
Type Strain:	yes
References:	5905: Krebs HA, Eggleston LV . Bacterial urea formation (Metabolism of Corynebacterium ureafaciens). Enzymologia 7: 310-320, 1939. 7828: Clark FE. The designation of Corynebacterium ureafaciens Krebs and Eggleston as Arthrobacter ureafaciens. (Krebs amd Eggleston) comb. nov.. Int. Bull. Bacteriol. Nomencl. Taxon. 5: 111-113, 1955. 9430: Dubos R, Miller BF . The production of bacterial enzymes capable of decomposing creatinine. J. Biol. Chem. 121: 429-445, 1937. 36887: Skerman VB, et al. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30: 225-420, 1980.

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Bacteria

ATCC® Number:	14756™ Order this item	**Price:**	**$145.00**
Organism:	*Serratia marcescens* Bizio		
Designations:	PCI 1107	**Isolation:**	Fort Detrick, MD, 1961 [80755]
Depositor:	FDA	**History:**	ATCC<<--FDA <<--Millipore Filter Corp.
Biosafety Level:	1	**Shipped:**	freeze-dried
Growth Conditions:	ATCC medium 3: Nutrient agar or nutrient broth Temperature: 26.0C		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		

Related Products

Cross References:	GenBank: U56906: Serratia marcescens DNA gyrase (gyrA) gene, complete cds.
Applications:	membrane filter testing [4087] [21611] sterility assurance [21611] testing antimicrobial agent [92595] testing antimicrobial handwashing formulations [4088] [4089] [32196] [92590]
References:	4087: ASTM International. Standard Test Method for Retention Characteristics of 0.40 to 0.45 mircrometer Membrane Filters Used in Routine Filtration Procedures for the Evaluation of Microbiological Water Quality. West Conshohocken, PA: ASTM International; ASTM Standard Test Method D 3863-87(Reapproved 2001). 4088: ASTM International. Standard Test Method for Evaluation of Health Care Personnel Handwash Formulation. West Conshohocken, PA: ASTM International; ASTM Standard Test Method E 1174-06. 4089: ASTM International. Standard Test Method for Evaluation of Antimicrobial Handwash Formulations by Utilizing Fingernail Regions. West Conshohocken, PA: ASTM International; ASTM Standard Test Method E 1327-07. 21611: U.S. Pharmacopeia. General Chapters: <1211> STERILIZATION AND STERILITY ASSURANCE OF COMPENDIAL ARTICLES. Rockville, MD: U.S. Pharmacopeia; USP USP28-NF23, 2005. 32196: Fendler EJ, et al. Antimicrobial cleansing composition containing chlorhexidine, an amphoteric surfactant, and an alkyl polyglucoside. US Patent 5,719,113 dated Feb 17 1998 92590: Standard Test Method for Determining the Bacteria-Eliminating Effectiveness

Exhibit E

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Bacteria

ATCC® Number:	**8363™** Order this item	**Price:**	**$225.00**
Organism:	*Desemzia incerta* (Steinhaus) Stackebrandt et al.; deposited as *Bacterium incertum* Steinhaus		
Designations:	[CIP 104227; DSM 20581; NCIB 9892]	**Isolation:**	ovaries of the lyreman cicada, Tibicen linnei
Depositor:	EA Steinhaus		
Biosafety Level:	1	**Shipped:**	freeze-dried
Growth Conditions:	ATCC medium : Rabbit blood agar Temperature: 30.0C		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		

Related Products

Cross References:	GenBank: Y17300: 16S rDNA sequence
Type Strain:	yes [5645] [36887] [38973]
Comments:	probably not a Brevibacterium [6296]
References:	5645: Steinhaus EA. A study of the bacteria associated with thirty species of insects. J. Bacteriol. 42: 757-790, 1941. 6296: Bergey's Manual Syst. Bacteriol. 2: 1309, 1986. 36887: Skerman VB, et al. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30: 225-420, 1980. 38973: Stackebrandt E, et al. Reclassification of Brevibacterium incertum (Breed 1953) as Desemzia incerta gen. nov., comb. nov.. Int. J. Syst. Bacteriol. 49: 185-188, 1999. PubMed: 10028261

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Exhibit F

Product Description

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Bacteria

ATCC® Number:	**14403™** Order this item	**Price:**	**$225.00**
Organism:	*Brevibacterium stationis* (ZoBell and Upham) Breed; deposited as *Achromobacter stationis* ZoBell and Upham		
Designations:	622		
Depositor:	CE ZoBell		
Biosafety Level:	1	**Shipped:**	freeze-dried
Growth Conditions:	ATCC medium 2: Marine agar 2216 or marine broth 2216 Temperature: 26.0C		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		
	Related Products		
Type Strain:	yes		
References:	8019: ZoBell CE, Upham HC . A list of marine bacteria including descriptions of sixty new species. Bull. Scripps Inst. Oceanogr. 5: 239-292, 1944. 36887: Skerman VB, et al. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30: 225-420, 1980.		

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Exhibit G

JSCC Catalogue of Cultures
Japan Society for Culture Collections

General information

Version 1.2.0 (Version history)

Order or Deposit Help for search

JSCC On-Line Database Search

Strain List

Scieictific Name: Brevibacterium fuscum Prior display:

JCM 1486
"*Brevibacterium fuscum*" (Zimmermann) Breed
←K. Suzuki CNF 194 ←AJ 1474 ←IFM AU-44.
=IFM AU-44
Cultivation: 30°C
Registered: 2008-01-14

JCM 1488
"*Brevibacterium fuscum*" (Zimmermann) Breed
←K. Suzuki CNF 196 ←AJ 3124 ←CCEB 277.
=CCEB 277 =IFO 12127 =NBRC 12127
Source: Silkworm (*Bombyx mori* Linnaeus)
Cultivation: 30°C
Registered: 2008-01-14

JSCC On-Line Database Search

Browser recommendation: Internet Explorer 6 or higher version, Netscape 6 or higher version.
Requirements: JavaScript and Cookie must be allowed in the browser.

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Secretariant: NITE Biological Resource Center (NBRC) E-mail

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Bacteria

ATCC® Number:	**482™** Order this item	**Price:**	**$225.00**
Organism:	*Cellulomonas flavigena* (Kellerman and McBeth) Bergey et al.		
Designations:	NRS 134 [BUCSAV 180; NCIB 8073; QM B-528]		
Depositor:	NR Smith		
Biosafety Level:	1	**Shipped:**	freeze-dried
Growth Conditions:	ATCC medium 3: Nutrient agar or nutrient broth Temperature: 30.0C		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		

Related Products

Type Strain:	yes
References:	10189: Zentralbl. Bakteriol., II Abt. 34: 485-494, 1912. 36887: Skerman VB, et al. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30: 225-420, 1980.

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Exhibit I

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Bacteria

ATCC® Number:	**21292™** Order this item **Price:** **$225.00**
Organism:	*Nocardia globerula* (Gray) Waksman and Henrici
Designations:	3909-92E
Depositor:	Kyowa Ferm. Ind. Co., Ltd.
Biosafety Level:	1 **Shipped:** freeze-dried
Growth Conditions:	ATCC medium 196: Yeast malt extract agar Temperature: 26.0C
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

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Related Products

Applications:	produces penicillin derivatives [2891] produces restriction endonuclease NgbI [51950]
References:	2891: Nara T, et al.. Process for producing penicillin derivatives. US Patent 3,682,777 dated Aug 8 1972 51950: Roberts RJ. List of known restriction endonucleases. *In:* New England Biolabs Catalog 1986/87. 1987, pp. 68-81.

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Exhibit J

Appl Microbiol Biotechnol
DOI 10.1007/s00253-005-0071-1

BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Yukie Akutsu-Shigeno · Yusuke Adachi · Chise Yamada · Kieko Toyoshima · Nobuhiko Nomura · Hiroo Uchiyama · Toshiaki Nakajima-Kambe

Isolation of a bacterium that degrades urethane compounds and characterization of its urethane hydrolase

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Abstract A bacterium which degrades urethane compounds was isolated and identified as *Rhodococcus equi* strain TB-60. Strain TB-60 degraded toluene-2,4-dicarbamic acid dibutyl ester (TDCB) and accumulated toluene diamine as the degradation product. The enzyme which cleaves urethane bond in TDCB was strongly induced by acetanilide. The purified enzyme (urethane hydrolase) was found to be homogeneous on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The molecular weight was estimated to be 55 kDa. The optimal temperature and pH were 45°C and 5.5, respectively. The enzyme hydrolyzed aliphatic urethane compound as well as aromatic ones. The activity was inhibited by $HgCl_2$, *p*-chrolomercuribenzoic acid, and phenylmethylsulfonyl fluoride, suggesting that cysteine and/or serine residues play an important role in the activity. The enzyme catalyzed the hydrolysis of anilides, amides, and esters as well as TDCB. It was characterized as a novel amidase/esterase, differing in some properties from other known amidases/esterases.

Abbreviations EPC: Ethyl *N*-phenylcarbamate · HDCB: Hexamethylene dicarbamic acid dibutyl ester · HDA: Hexamethylene diamine · HFBA: Heptafluorobutyric acid anhydride · MDCB: Methylene bisphenyl dicarbamic acid dibutyl ester · PMSF: Phenylmethylsulfonyl fluoride · PUR: Polyurethane · TDA: Toluene diamine · TDI: Tolylene diisocyanate

Y. Akutsu-Shigeno · K. Toyoshima · T. Nakajima-Kambe
SORST, Japan Science and Technology Agency,
4-1-8 Honcho Kawaguchi,
Saitama, Japan

Y. Akutsu-Shigeno · Y. Adachi · C. Yamada · N. Nomura · H. Uchiyama · T. Nakajima-Kambe
Graduate School of Life and Environmental Science,
University of Tsukuba,
Tsukuba, Ibaraki, 305-8572, Japan

T. Nakajima-Kambe (✉)
Institute of Applied Biochemistry, University of Tsukuba,
Tsukuba, Ibaraki, 305-8572, Japan
e-mail: toshi@sakura.cc.tsukuba.ac.jp
Tel.: +81-29-8534619
Fax: +81-29-8534605

Introduction

Inasmuch as environmental pollution by plastic wastes has become a serious issue, polyurethane (PUR) is expected to be biodegradable in a particular field. PUR is generally resistant to microbial attack, but polyester-based PUR is susceptible to degradation and some degrading bacteria and fungi have been isolated (Nakajima-Kambe et al. 1999). There are a few reports concerning the characterization of PUR-degrading enzymes; however, most enzymes hydrolyzed only ester bonds in the PUR (Akutsu et al. 1998; Howard 2002). Martens and Domsch (1981) detected a very limited amount of degradation products derived from the hydrolysis of urethane bonds after a 3-month incubation of PUR foams in various media. Santerre et al. (1994) investigated the biodegradation of PUR using hydrolytic and oxidative enzymes. They suggested that urethane bonds were not cleaved and remained in the degradation products. We have reported that low-molecular-weight urethane compounds remained after the microbial degradation of PUR (Nakajima-Kambe et al. 1997). For complete degradation of PUR, the cleavage of the urethane bond is necessary.

Urethane compounds, represented by the general formula R_1-NHCOO-R_2, are classified based on their types of substitutions. Three types of hydrolases which cleave the urethane bond have been well studied: (I) alkyl carbamate hydrolase (R_1, H; R_2, alkyl group), (II) *N*-alkyl arylcarbamate hydrolase (R_1, alkyl group; R_2, aryl group), and (III) *N*-aryl acylcarbamate hydrolase (R_1, aryl group; R_2, alkyl group). A few alkyl carbamate hydrolases (type I) that hydrolyze ethyl carbamate to ethanol and ammonia were identified (Kobashi et al. 1990; Zhao and Kobashi 1994). There are many reports on bacteria expressing type II hydrolases. In particular, assimilation of carbaryl and carbofuran, which are used as pesticides and herbicides, has been extensively studied, and several enzymes hydro-

lyzing them were identified and investigated (Derbyshire et al. 1987; Karns and Tomasek 1991; Mulbry and Eaton 1991; Hayatsu and Nagata 1993; Hayatsu et al. 2001). Phenylcarbamate hydrolases are well-known examples of type III hydrolases and characterized as amidase or esterase (Marty and Vouges 1987; Pohlenz et al. 1992).

Most of the urethane compounds previously studied are type I and type II substrates, whereas urethane groups in the most common type of PUR, synthesized with TDI, are type III substrates. Owen et al. (1996) focused on the cleavage of the urethane bond in PUR. They used low-molecular-weight *N*-tolylcarbamate esters and identified metabolites derived from the hydrolysis of the urethane bond. The bacterium, *Exophiala jeanselmei* REN-11A, degraded the substrate forming toluene diamine (TDA) as the hydrolysis product. Unfortunately, no information on its hydrolase is available.

To obtain useful information on the hydrolysis of the urethane group in the TDI-based PUR, we investigated the biodegradation of a TDI-based urethane compound of low molecular weight. In this study, we isolated a bacterium that is capable of utilizing the urethane compound, toluene-2,4-dicarbamic acid dibutyl ester (TDCB). Furthermore, the properties of the enzyme, named as urethane hydrolase, which cleaves the urethane bond are discussed.

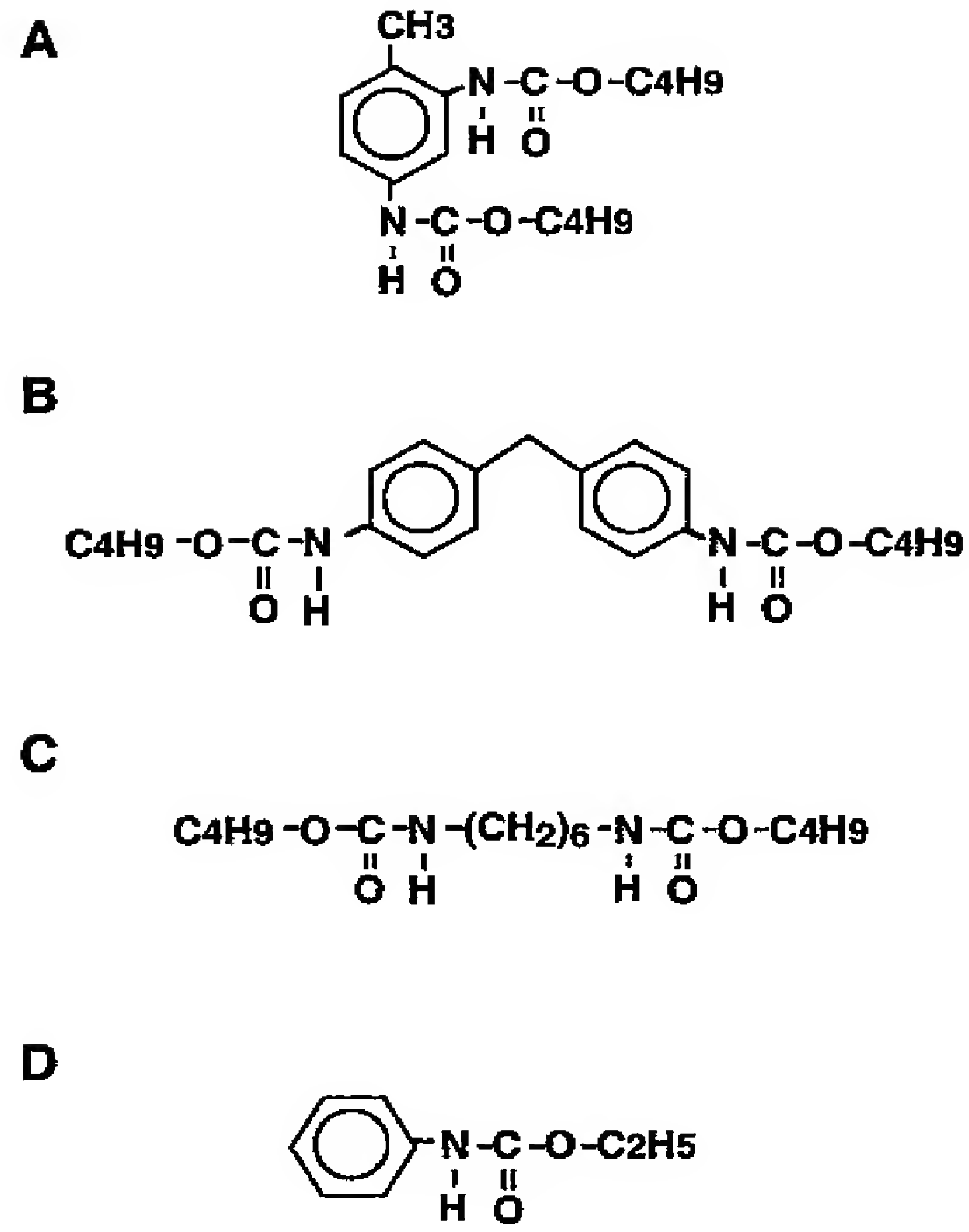

Fig. 1 Urethane compounds used in this study. **a** TDCB, **b** MDCB, **c** HDCB, and **d** EPC

Materials and methods

Materials

The urethane compounds TDCB, methylene bisphenyl dicarbamic acid dibutyl ester (MDCB), and hexamethylene dicarbamic acid dibutyl ester (HDCB) were provided by Mitsui Takeda Chemicals Co., Ltd. (Tokyo, Japan) (Fig. 1). All the other compounds used were from commercial sources.

Media

The mineral medium used for the screening of soil bacteria contained the following (per liter of distilled water): KH_2PO_4, 2.0 g; K_2HPO_4, 7.0 g; NH_4NO_3, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g, $FeSO_4 \cdot 7H_2O$, 10 mg; $ZnSO_4 \cdot 7H2O$, 1.0 mg; $CuSO_4 \cdot 7H_2O$, 0.01 mg; and $MnSO_4 \cdot 6H_2O$, 2.0 mg. For the growth of *Rhodococcus equi* TB-60, vitamins were supplemented to the mineral medium as follows (per liter of mineral medium): nicotinamide, 10.0 mg; pantothenic acid, 2.5 mg; thiamine, 2.5 mg; riboflavin, 1.25 mg; pyridoxine, 0.75 mg; *p*-aminobenzoic acid, 0.6 mg; folic acid, 0.5 mg; and biotin, 0.1 mg.

Bacterial isolation and identification

Soil samples collected from various sites in Japan were inoculated into the mineral medium containing 3 mM TDCB as the sole source of carbon and energy and incubated at 30°C in a reciprocal shaker (120 oscillations/min). Subcultures were carried out four times by taking 0.5 ml of the original culture and inoculating it into 10 ml of fresh medium. The enrichment culture broth was spread on nutrient broth (Difco Laboratory, MI, USA) agar plates. Isolates that grew on the medium were harvested and stored at −80°C. For the identification of the isolates, 16S rRNA gene sequencing was performed. The full-length gene was amplified by PCR, and the nucleotide sequences of parts of the 5′ and 3′ regions were determined. DNA sequencing was carried out using an ABI Prism 310 DNA sequencer (PE Biosystems, Tokyo, Japan). Comparison with other 16S rRNA gene sequences was performed using the BLAST program (Altschul et al. 1990) against the GenBank database.

Quantitative analysis of TDCB metabolites

The amount of TDCB in the culture broth was determined by high-performance liquid chromatography (HPLC). An equal volume of acetonitrile was added to the culture broth, and cells were removed by centrifugation. The supernatant was injected to an HPLC system (GL Science, Tokyo, Japan) equipped with a TSK-GEL ODS-80 column (Tosoh, Tokyo, Japan) and a UV detector (JASCO, Tokyo, Japan). The conditions for HPLC were described previously (Nakajima-Kambe et al. 1997). The amount of TDA was measured by gas chromatography (GC). The culture broth was extracted with ethyl acetate and injected into a gas

chromatograph (Shimadzu, Kyoto, Japan) equipped with a DB-1 column (30-m length, 0.25-mm inside diameter, 0.25-μm film thickness; J&W Scientific, USA) and a flame ionization detector. The temperatures of the injector, column, and detector were 300, 200, and 300°C, respectively.

Enzyme production

R. equi TB-60 cultivated on a nutrient broth (Difco) agar plate for 2 days was collected and inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the mineral medium supplemented with vitamins and 0.1 g of butylcarbamate as the sole carbon source. The flask was shaken on a rotary shaker for 24 h at 30°C. Then, the culture broth was transferred into a 3-l Erlenmeyer flask containing 1,000 ml of mineral medium supplemented with vitamins, 1.0 g of butylcarbamate, and 1.0 g of acetanilide. The cells were harvested after cultivation for 36 h at 30°C.

Enzyme activity assays

Urethane hydrolase activity

(a) Reaction mixtures contained 0.2 ml of 0.1% (w/v, in ethanol solution) ethyl *N*-phenylcarbamate (EPC), 0.7 ml of 0.1 M potassium phosphate buffer (pH 7.0), and 0.1 ml of the enzyme solution. The reaction was carried out at 30°C for 10 min and stopped by adding 0.1 ml of 6N HCl to the reaction mixture. The amount of aniline formed in the reaction mixture was measured by the diazotation method (Heyman et al. 1981). One unit is defined as the amount of enzyme required to liberate 1 μmol of aniline per minute.

TDCB hydrolyzing activity

(b) Reaction mixtures contained 0.01 ml of 1.0% (w/v, in ethanol solution) TDCB, 0.89 ml of 0.1 M potassium phosphate buffer (pH 7.0), and 0.1 ml of the enzyme solution. The reaction was carried out at 30°C for 6 h, and residual TDCB was measured as described above.

Enzyme purification

Throughout the purification procedure, EPC was used as a substrate to measure enzyme activity. Cells (approximately 14 g wet cells) were suspended in 30 ml of 20 mM potassium phosphate buffer (pH 7.0) and disrupted using a French Pressure Cell (SIM Aminco, NY, USA) at a pressure of 1,280 psi three times. The treated cell suspension was centrifuged at 20,000 rpm for 30 min to remove cell debris. The supernatant was fractionated with ammonium sulfate (40–60% saturation), and the precipitate was dissolved in 20 mM potassium phosphate buffer (pH 7.0). The solution was desalted using a HiTrap Desalting column (Amersham Bioscience, Uppsala, Sweden) equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol (DTT). The desalted fractions were collected and applied to a Resource Q column (6.0-ml bed volume; Amersham Bioscience) equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT at a flow rate of 2.0 ml/min. After washing the column with the same buffer, the enzyme was eluted by linearly increasing the ionic strength of NaCl over the range of 0–0.4 M/60 min in the same buffer. The active fractions were collected and concentrated by ultrafiltration (YM10, Millipore, MA, USA). The purified enzyme was stored in 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT and 20% glycerol at 4°C until use.

Protein determination

Protein concentration was determined following the method of Lowry et al. (1951). Bovine serum albumin was used as the standard.

Molecular weight determination

The native size of the purified enzyme was determined by gel filtration on a Superose 12 HR10/30 column (Amersham Bioscience) as described previously (Akutsu et al. 1998). The subunit size of the enzyme was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970).

Electrophoresis and immunoblotting

After SDS-PAGE, the proteins on the gel were electroblotted onto polyvinylidene difluoride membranes for the detection of the urethane hydrolase. The blots were incubated with rabbit antiserum, raised against the urethane hydrolase, followed by incubation with an alkaline phosphatase conjugated sheep antirabbit IgG (CHEMICON, CA, USA) and developed by using nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (Roche Diagnostics, Mannheim, Germany).

Identification of hydrolysis products of urethane compounds

The reaction mixtures contained 0.8 ml of 0.1 M potassium phosphate buffer (pH 7.0) and 0.1 ml of a 0.1% (w/v) ethanol solution of TDCB, MDCB, and HDCB. The reaction was initiated by the addition of 0.1 ml of the enzyme solution. After a 3-h incubation at 30°C, the products were extracted with ethyl acetate and analyzed by GC–mass spectrometry (GC-MS). For the reaction of HDCB, the products were extracted with toluene under alkaline con-

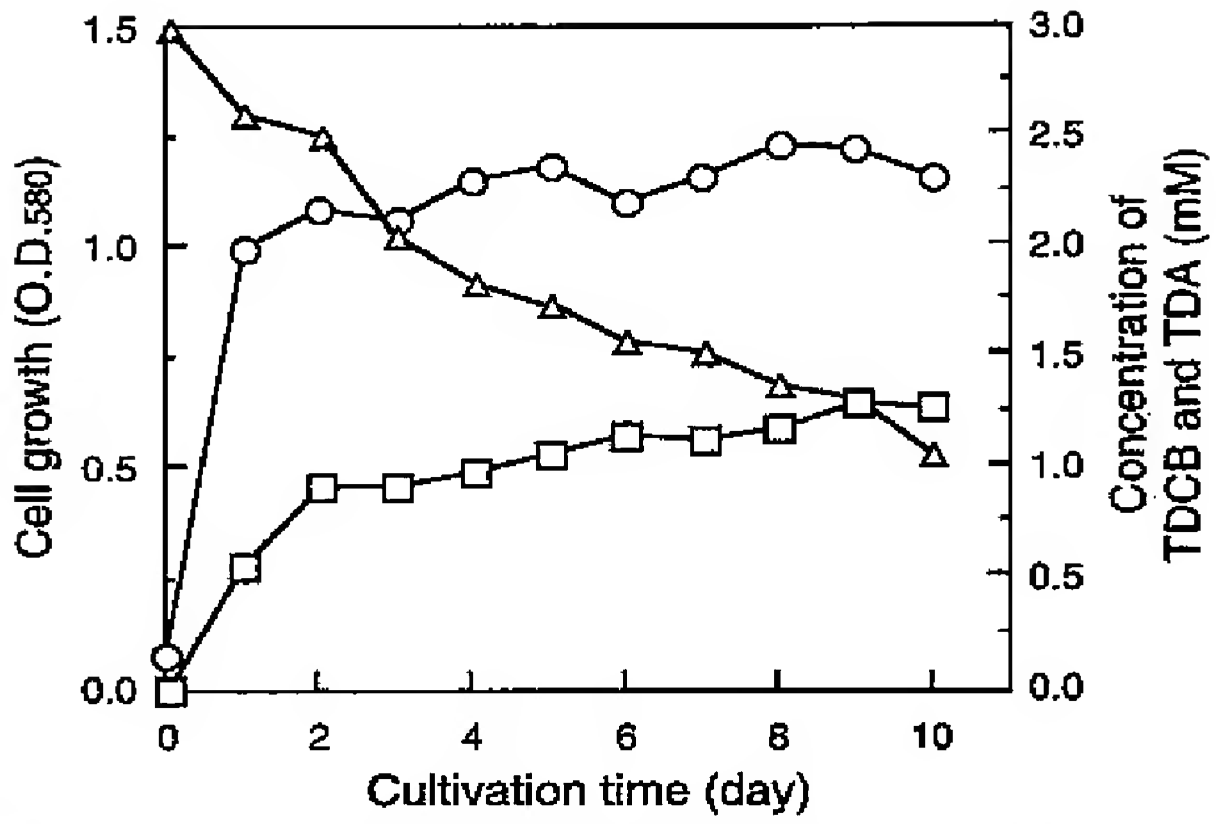

Fig. 2 Growth of *R. equi* TB-60 in the mineral medium with TDCB. *R. equi* TB-60 was inoculated into the mineral medium with 3 mM TDCB as the sole carbon source. The concentrations of TDCB (*triangles*) and TDA (*squares*) in the culture broth and optical density at 580 nm (OD 580) (*circles*) were measured as described in Materials and methods

ditions and subsequently derivatized using heptafluorobutyric acid anhydride (HFBA) following the method of Skarping et al. (1988).

Substrate specificity

Substrate specificity was examined in the reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.0), 10 mM substrate, and the appropriate amount of the enzyme solution. The reaction was carried out at 30°C for 10 min. The amount of ammonia released from amide and carbamate derivatives, except for EPC, was determined by the modified indophenol method using Ammonia-Test Wako (Wako Pure Chemicals, Osaka, Japan). The amount of aniline released from acetanilide and EPC was determined by the diazotation method (Heyman et al. 1981). The activities toward *p*-nitroanilide derivatives and *p*-nitrophenyl esters were spectrophotometrically determined following the method of Kay et al. (1993). The kinetic parameters were determined from Lineweaver–Burk plots of enzyme activity by using a least-squares best fit of the Michaelis–Menten equation.

Results

Isolation and identification of TDCB-degrading bacteria

Approximately 350 soil samples were screened for TDCB-utilizing bacteria by enrichment culture using TDCB as the sole carbon source. One bacterium, designated strain TB-60, was isolated from the enrichment culture. Strain TB-60 was an aerobic, Gram-positive bacterium showing pleomorphism. It formed smooth, shiny, white colonies and produced abundant slime. The sequence analysis of 515 bases at the 5′ end and 484 bases at the 3′ end of the 16S rRNA gene of strain TB-60 showed 99 and 100% identities, respectively, with those of the typical *R. equi* in the BLAST database. Inasmuch as strain TB-60 was in accordance with morphological and taxonomical characteristics of the type strain of *R. equi*, except for colony color (Goodfellow 1986), it was identified as *R. equi*.

Time course of TDCB degradation by strain TB-60

Figure 2 shows the time courses of cell growth and the concentration of TDCB and TDA during the cultivation of

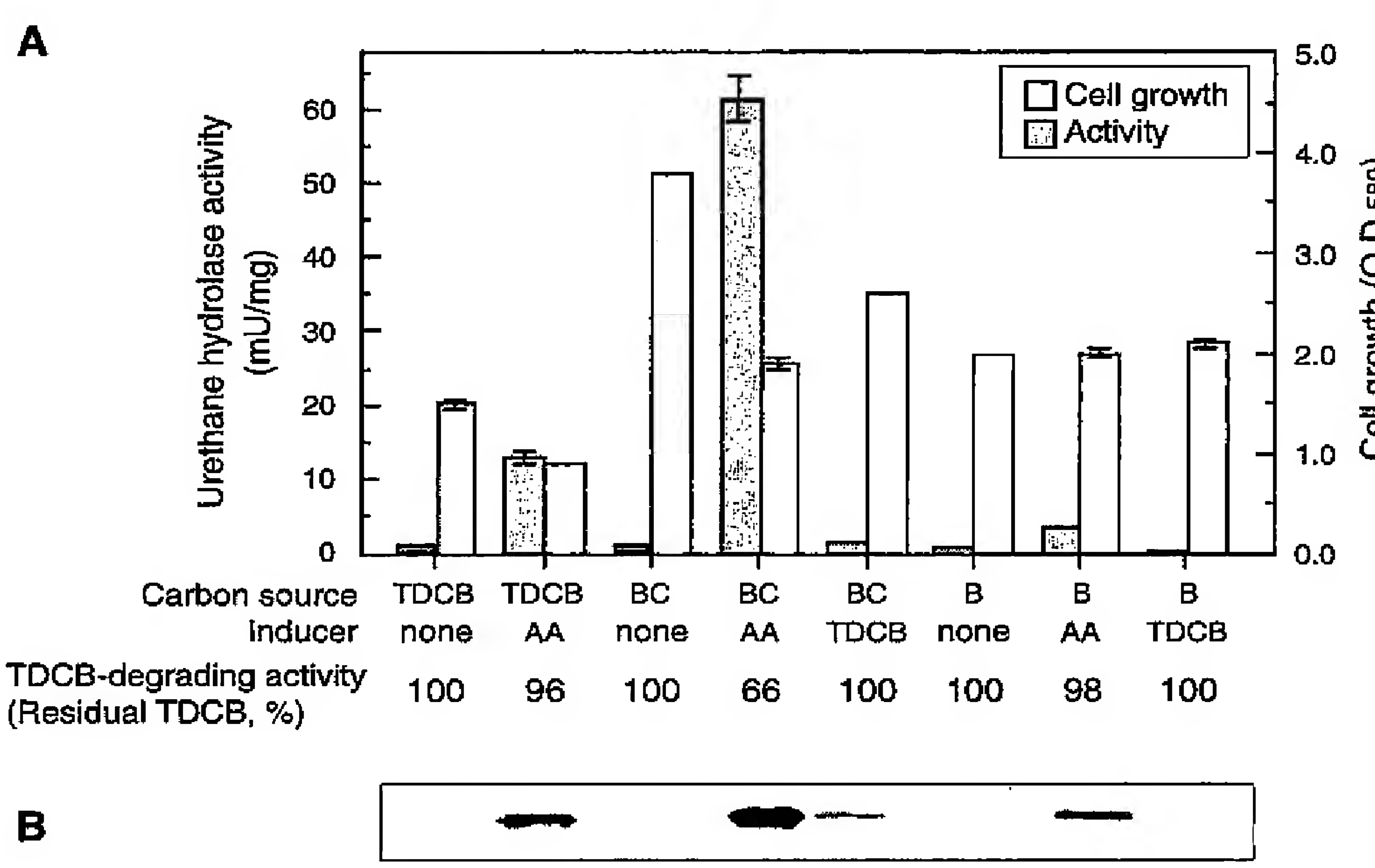

Fig. 3 Effects of carbon sources and inducers on growth and enzyme activity of *R. equi* TB-60. Strain TB-60 was inoculated into the mineral media containing 0.1% of carbon sources and 0.1% of inducers and cultured at 30°C for 2 days with shaking at 120 rpm. Cell-free extracts were obtained by the disruption of the cells using a sonicator. **a** Cell growth and urethane hydrolase activity of the cell-free extract. TDCB-degrading activity was measured as described in Materials and methods. *BC* Butylcarbamate, *B* butanol, *AA* acetanilide. **b** Western blotting of the urethane hydrolase in the cell-free extract. A total of 2.5 μg of each protein was used for electrophoresis and analyzed by Western blotting using anti-urethane hydrolase antibodies

strain TB-60 using 3 mM TDCB as the sole carbon source. When strain TB-60 was not inoculated, neither the decrease in TDCB nor TDA accumulation was observed. After a 2-day cultivation, moderate cell growth and TDA accumulation were observed in conjunction with a decrease in the amount of TDCB, resulting in 70% of the TDCB being degraded over 10 days. Inasmuch as strain TB-60 could utilize butanol but not TDA as its sole carbon source (data not shown), it appears that this strain utilizes butanol derived from TDCB by the cleavage of urethane bonds.

Induction of urethane hydrolase

The cell-free extracts prepared from TB-60 cells which grew in media containing various carbon sources and inducers were tested for their urethane hydrolase and TDCB-degrading activities (Fig. 3). In the culture supernatant, no activity was detected under any culture conditions. When TDCB was used as the sole carbon source, TDCB-degrading activity was below the detection limit. TDCB is not a suitable substrate to induce production of the enzyme because it is almost totally insoluble in water resulting in poor growth and is not available in sufficient quantity to be used for large-scale culture. When butanol or butylcarbamate was used as the carbon source, TB-60 showed good cell growth. The addition of acetanilide, a TDCB analogue with an amide instead of a urethane bond, led to an increase in the urethane hydrolase and TDCB-degrading activities. The induction effect was marked when butylcarbamate was used as the carbon source.

Purification and properties of urethane hydrolase

The highest urethane hydrolase activity was detected for the cultivation period of 36 h in the medium containing 0.1% acetanilide. The urethane hydrolase was effectively purified via two simple steps, ammonium sulfate fractionation and Resource Q column chromatography, resulting in a 58% yield and a specific activity of 6.0 U/mg. The addition of glycerol at 20% was necessary upon storage of

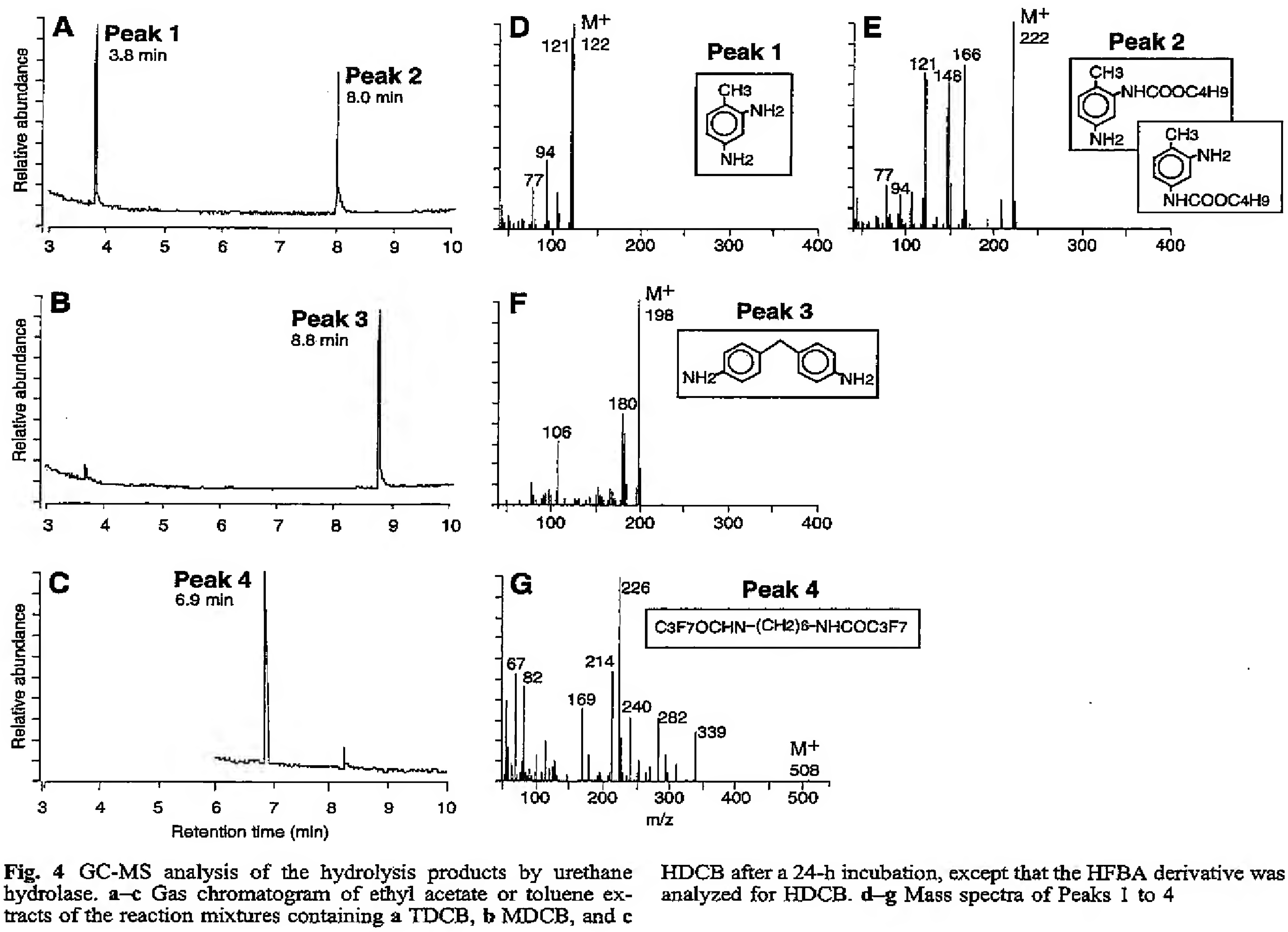

Fig. 4 GC-MS analysis of the hydrolysis products by urethane hydrolase. **a–c** Gas chromatogram of ethyl acetate or toluene extracts of the reaction mixtures containing **a** TDCB, **b** MDCB, and **c** HDCB after a 24-h incubation, except that the HFBA derivative was analyzed for HDCB. **d–g** Mass spectra of Peaks 1 to 4

the enzyme at 4°C. The purified enzyme showed only one protein band with a subunit molecular weight of 55 kDa on SDS-PAGE. The molecular weight of the native urethane hydrolase was also estimated to be approximately 55 kDa by gel filtration. These observations indicate that this enzyme is a monomer.

Hydrolysis of urethane compounds

Purified urethane hydrolase was tested for its ability to hydrolyze some urethane compounds. The products of hydrolysis were analyzed and identified by GC-MS (Fig. 4). When TDCB was used as the substrate, two peaks corresponding to hydrolysis products were detected on GC. The mass spectrum of peak 1 with a retention time of 3.8 min was identical to that of authentic TDA. The spectrum of peak 2 was different from that of either TDCB or any other chemicals in the NIST mass spectral database but was characteristic of carbamic acid (amino methylphenyl) butyl ester (Fig. 4e). This product was also detected in the culture broth of TB-60 when TDCB was used as the sole carbon source (data not shown). The isomers could not be separated under the GC conditions. When MDCB was used as the substrate, a single-product peak (peak 3) was detected. A comparison of the mass spectrum of peak 3 to that of the authentic compound indicated that this product is 4,4′-diaminodiphenyl methane (Fig. 4f). Inasmuch as aliphatic amines are difficult to analyze by standard GC, the derivatization of a free amino group to an amide is necessary (Skarping et al. 1988). For the identification of the hydrolysis product of HDCB, an HFBA derivative of the product was analyzed by GC-MS (Fig. 4g). A single peak (peak 4) was detected on GC, and its spectrum was identical to that of the HFBA derivative of authentic hexamethylene diamine (HDA). This result indicates that the hydrolysis product of HDCB is HDA.

Table 1 Effect of various compounds on urethane hydrolase activity

Compound	Relative activity (%)
None	100
$CdCl_2$	82±0
$CoCl_2$	89±0
$CuSO_4$	39±1.7
$HgCl_2$	2±0.09
$MgCl_2$	120±3.5
$MnCl_2$	96±0
$NiSO_4$	68±1.7
$ZnSO_4$	79±1.7
EDTA	98±1.4
8-Hydroxyquinoline	99±1.9
DTT	91±1.4
p-Chloromercuribenzoic acid	63±2.7
Iodoacetamide	94±1.9
N-Ethylmaleimide	112±1.9
PMSF	16±0.9

The standard enzyme assay was performed except for the addition of various compounds at a concentration of 1 mM

Table 2 Substrate specificity of urethane hydrolase

Substrate	Relative activity[a]	K_m (mM)	k_{cat}/K_m (mM^{-1} sec^{-1})
Anilide			
Acetanilide	36.5	0.13	86.5
p-Nitroacetanilide	9.2	0.15	79.4
L-Alanine-*p*-nitroanilide	0.29	2.49	1.8
L-Leucine-*p*-nitroanilide	Nd	–	–
Carbamate			
Ethyl carbamate	0.11	6.59	<0.01
Butyl carbamate	0.003	Nt	Nt
EPC	1	0.07	7.5
Amide			
Acetamide	1.7	49.0	0.14
Butyramide	10.4	2.5	4.7
Benzamide	0.25	11.8	<0.01
Phenyl acetamide	0.069	9.4	<0.01
Ester			
p-Nitrophenyl acetate	21.9	0.07	287.3
p-Nitrophenyl butyrate	16.7	0.10	175.8
Methyl benzoate	Nd	–	–
Ethyl benzoate	Nd	–	–

Nd Not detected, *Nt* not tested
[a]The specific activity toward EPC was taken as one. Enzyme activities were measured as described in Materials and methods

Optimal conditions

The effects of temperature and pH on the enzyme activity and stability were determined. The optimal temperature and pH were 45°C and 5.5, respectively. Almost all the activity was lost with the incubation of the enzyme in the buffer for 30 min at 40°C. However, no decrease in the activity was observed when the enzyme was incubated with 10 mM EPC for 30 min at 40°C. Under this condition, the incubation for 30 min at 60°C is necessary to inactivate the enzyme completely. The enzyme was stable over the pH range of 8–10.

Effect of chemicals

The effects of potential inhibitors are shown in Table 1. The urethane hydrolase activity was not influenced by most metals, except for $HgCl_2$ and $CuSO_4$, or by metal chelators such as ethylenediaminetetraacetic acid (EDTA) and 8-hydroxyquinoline. The enzyme was inhibited by $HgCl_2$ and *p*-chloromercuribenzoic acid but neither iodoacetamide nor *N*-ethylmaleimide. Phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, also inhibited the enzyme activity.

Substrate specificity

The purified urethane hydrolase showed a wide substrate specificity toward anilides, amides, carbamate, and esters (Table 2). Acetanilide and *p*-nitroacetanilide were effectively hydrolyzed but not the *p*-nitroanilide derivatives of amino acids, such as L-alanine and L-leucine. Among the carbamates tested, substrates with a free carbamoyl group, such as ethyl carbamate and butyl carbamate, were scarcely hydrolyzed. High hydrolytic activities toward *p*-nitrophenyl esters were also observed with a high catalytic efficiency (k_{cat}/K_m); however, benzyl esters were not hydrolyzed. The enzyme showed hydrolytic activities toward aliphatic amides, acetamide, and butyramide but lower activities toward aromatic amides. The specificity constants of these aliphatic amides were much lower than those of anilides or nitrophenyl esters.

Discussion

A bacterium that utilizes TDCB as the sole carbon source was isolated and identified as *R. equi*. The accumulation of TDA as the TDCB metabolite indicates the hydrolysis of the urethane bonds in TDCB. Strain TB-60 degraded 1.5 mM TDCB in 7 days. It is superior in this respect to *E. jeanselmei* REN-11A, which degraded 0.3 mM toluene-2, 6-dicarbamic acid diethyl ester in 7 days (Owen et al. 1996). The mass imbalance of the TDA produced compared with TDCB consumed appears to be caused by the accumulation of an intermediate, carbamic acid (amino methylphenyl) butyl ester. This intermediate was detected in the culture broth as well as in the enzymatic reaction mixture (Fig. 4).

Inasmuch as TB-60 could utilize butanol (Fig. 3) but not TDA as the carbon source, it appears that TB-60 utilizes butanol, which is the hydrolysis product of TDCB. The growth of TB-60 on TDCB is lower than that on butanol (Fig. 3). It is assumed that the extremely low solubility of TDCB in water causes the limited supply of the carbon source. As the growth of TB-60 was inhibited by TDA at concentrations higher than 1.0 mM (data not shown), TDA accumulation following TDCB hydrolysis may lead to the inhibition of the growth after 2 days of cultivation (Fig. 2). Furthermore, TDCB-degrading activity was not detected in the cells grown on TDCB. Such low enzyme activity, below the detection limit, may have caused the slow growth of TB-60 in the medium with TDCB.

For the production of urethane hydrolase, a more suitable carbon source and inducer than TDCB should be used. As shown in Fig. 3, the medium containing butylcarbamate and acetanilide showed extremely high TDCB-degrading activity as well as urethane hydrolase activity. Inasmuch as no significant activity was detected in the medium containing only butylcarbamate, the role of acetanilide must be as an inducer of a TDCB-degrading enzyme. Interestingly, the Western blot analysis revealed that the expression of the urethane hydrolase was also induced by TDCB when butylcarbamate was used as the carbon source, even though its TDCB-degrading activity was too weak to detect (Fig. 3). Furthermore, purified urethane hydrolase could hydrolyze TDCB, producing TDA as the final product. These results demonstrate that the enzyme induced by acetanilide is a TDCB-degrading enzyme.

The urethane compounds used in this study are composed of isocyanate (R-NCO) and butanol linked through a urethane bond. Among the isocyanates used in PUR synthesis, TDI and methylene bisphenyl isocyanate are the most common (Nakajima-Kambe et al. 1999). The urethane hydrolase of *R. equi* TB-60 hydrolyzes the urethane bonds in TDCB, MDCB, and HDCB, forming the corresponding amines. Concerning the degradation of urethane compounds related to TDI-based PUR, only *E. jeanselmei* strain REN-11A is known to degrade toluene-2,4-dicarbamic acid diethyl ester (Owen et al. 1996). Unfortunately, the degradation activities toward any other substrates were not investigated, and the enzyme related to the degradation is still unknown. This is the first report on the characterization of an enzyme that hydrolyzes urethane bonds in substrates related to the urethane segment in PUR.

Concerning the active amino acids, the enzyme was sensitive to thiol-specific inhibitors, $HgCl_2$ and *p*-chrolomercuribenzoic acid, as well as serine protease inhibitor, PMSF. The same effects were observed for the esterase and amidase activities; *p*-nitrophenyl acetate and *p*-nitroacetanilide were used as the substrates, respectively (data not shown). These results suggest that cysteine and/or serine residues play an important role in the activity, and the catalytic mechanisms toward substrates with different linkages, i.e., urethane, ester, and amide, are similar. It is noted that the enzyme was not inhibited by thiol modifiers, *N*-ethylmaleimide and iodoacetamide. It is assumed that these reagents were less accessible to the active amino acids than $HgCl_2$ and *p*-chrolomercuribenzoic acid.

Inasmuch as compounds with urethane bonds are rarely found in the natural environment, it is unlikely that the enzyme is specialized to hydrolyze urethane bonds. There are some reports on the characterization of phenylcarbamate hydrolase with esterase or amidase activity (Engelhardt et al. 1973; Alt et al. 1975; Marty and Vouges 1987; Pohlenz et al. 1992). The hydrolase from *Pseudomonas alcaligenes* degraded phenylcarbamate herbicides as well as acylanilide, suggesting that it is an amidase (Marty and Vouges 1987). The substrate specificity and the nucleotide sequence of the phenylcarbamate hydrolase gene from the phenmedipham-degrading bacterium, *Arthrobacter oxydans* P52, revealed that it was carboxylesterase (Pohlenz et al. 1992). The observations, that the urethane hydrolase from *R. equi* TB-60 is strongly induced by acetanilide and exhibits high-specific activities toward anilides, indicate that this enzyme is likely to be an aryl acylamidase. However, the comparison of the k_{cat}/K_m values indicates that the reactivity toward phenylesters is markedly higher than that toward any other substrates tested. Furthermore, the enzyme preferred acidic conditions, differing from the known bacterial aryl acylamidases, the optimum pHs of which are in the alkaline range between 8 and 10 (Alt et al. 1975; Hammond et al. 1983; Yoshioka et al. 1991; Shimizu

et al. 1992). From these results, the urethane hydrolase of TB-60 was determined to be a novel amidase/esterase differing in some properties from other known amidases/esterases. It is estimated that the enzyme recognizes the substrates in which the carbamate or amide groups are substituted by aryl groups. For the ester substrates, similarly, the substitution to aryl groups is important for the activity because the substrates with benzoyl groups were not hydrolyzed. To understand the catalytic mechanism of this enzyme and intensify the cleavage activity for the urethane bond, the molecular cloning of the corresponding gene is in progress.

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